

## **Analysing the effect of I1 imidazoline receptor ligands on DSS-induced acute colitis in mice**

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## **Abstract**

Imidazoline receptors (IRs) have been recognized as promising targets in the treatment of numerous diseases, and moxonidine and rilmenidine, agonists of I<sub>1</sub>-IRs are widely used as antihypertensive agents. Some evidence suggests that IR ligands may induce anti-inflammatory effects acting on I<sub>1</sub>-IRs or other molecular targets, which could be beneficial in patients with inflammatory bowel disease (IBD). On the other hand, several IR ligands may stimulate also alpha<sub>2</sub>-adrenoceptors, which was earlier shown to inhibit, but in more recent studies to rather aggravate colitis. Hence, this study aimed to analyse for the first time the effect of various I<sub>1</sub>-IR ligands on intestinal inflammation. Colitis was induced in C57BL/6 mice by adding dextran sulfate sodium (DSS) to the drinking water for 7 days. Mice were treated daily with different IR ligands; moxonidine and rilmenidine (I<sub>1</sub>-IR agonists), AGN 192403 (highly selective I<sub>1</sub>-IR ligand, putative antagonist), efaroxan (I<sub>1</sub>-IR antagonist), as well as with the endogenous IR agonists agmatine and harmane. It was found that moxonidine and rilmenidine at clinically relevant doses, similarly to the other IR ligands, do not have a significant impact on the macroscopic and histological signs of DSS-evoked inflammation. Likewise, colonic myeloperoxidase and serum interleukin-6 levels remained unchanged in response to these agents. Thus, our study demonstrates that imidazoline ligands do not influence significantly the severity of DSS-colitis in mice and suggest that they probably neither affect the course of IBD in humans. However, the translational value of these findings needs to be verified with other experimental colitis models and human studies.

**Keywords:** colitis, inflammatory bowel disease, dextran sulfate sodium, imidazoline receptor

## 1. Introduction

The discovery of non-adrenergic binding sites that may mediate the hypotensive effect of clonidine and other imidazoline derivatives can be dated back to the 1980s (Bousquet et al. 1984). Since then pharmacological studies have revealed the existence of at least three distinct types of imidazoline receptors (IRs), named I<sub>1</sub>-, I<sub>2</sub>- and non-I<sub>1</sub>-non-I<sub>2</sub> or I<sub>3</sub>-IRs, and suggested that they may be promising targets in the treatment of numerous diseases, including hypertension, diabetes, depression and chronic pain (for reviews see e.g. Bousquet et al. 2003; Eglen et al. 1998; Head and Mayorov 2006; Li and Zhang 2011). Research on IRs has led to the development of moxonidine and rilmenidine, which have higher affinity for I<sub>1</sub>-IRs than for alpha<sub>2</sub>-adrenoceptors and used world-wide as antihypertensive agents (Edwards et al. 2012; Verbeuren et al. 1990). Moreover, it has also led to an intensive search for their endogenous ligands, and various substances were proposed as likely candidates, such as clonidine displacing substance (CDS) (Atlas and Burstein 1984), agmatine (Li et al. 1994), harmaline (Parker et al. 2004) and imidazole-acetic acid ribotide (Prell et al. 2004).

Concerning the gastrointestinal (GI) tract, radioligand binding studies provided evidence for the presence of IRs in the stomach and intestines of different mammals (Houi et al. 1987; Molderings et al. 1998; Senard et al. 1990; Wikberg et al. 1991). CDS and agmatine were also identified in the GI tract (Meeley et al. 1992; Raasch et al. 1995), in fact, the highest concentrations of agmatine were measured in the stomach and intestines among all tissues in the rat (Raasch et al. 1995). Because these data suggest an important role for IRs and their ligands in the gut, in the last two decades a number of studies were conducted to assess the GI effects of various non-selective and highly selective I<sub>1</sub>- and I<sub>2</sub>-IR ligands, sometimes leading to conflicting results (e.g. Colucci et al. 1998; Glavin and Smyth 1995; Kaliszan et al. 2006; Liu and Coupar 1997; Zádori et al. 2013).

Some sporadic evidence suggests that imidazoline drugs may induce anti-inflammatory effects as well. For example, clonidine and its structural analog (Holsapple et al. 1984; Kulkarni et al. 1986) as well as rilmenidine (Gyires et al. 2009) inhibited acute paw edema in rats, which was likely mediated by  $\alpha_2$ -adrenoceptors (Gyires et al. 2009; Kulkarni et al. 1986). Moxonidine reduced the level of the pro-inflammatory cytokine tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) in the serum of hypertensive postmenopausal women (Pöyhönen-Alho et al. 2008). Furthermore, some data suggest that  $I_1$ -IR ligands may interfere with sphingosine 1-phosphate (S1P) receptor signaling (Molderings et al. 2007a, b). Because S1P receptors have pivotal role in the regulation of immune cell trafficking (reviewed e.g. by Aoki et al. 2016; Chi 2011; Cyster and Schwab 2012), it can be speculated that  $I_1$ -IR ligands may have immunomodulatory properties, similarly to the S1P receptor modulator FTY720, which has potent anti-inflammatory effect in various clinical and preclinical inflammatory conditions, including dextran sulfate sodium (DSS)- and 2,4,6-trinitrobenzenesulfonic acid (TNBS)-evoked murine colitis (Daniel et al. 2007; Deguchi et al. 2006). In short, these data suggest that imidazoline drugs, such as moxonidine and rilmenidine, may have beneficial (and yet unrecognized) effects in patients with inflammatory diseases, including colitis.

On the other hand, many IR ligands can bind to and activate  $\alpha_2$ -adrenoceptors as well (Szabó 2002). This might contribute to their anti-inflammatory effect in some types of inflammation (see above), and according to earlier studies may improve the symptoms of patients with inflammatory bowel disease (IBD) as well (Furlan et al. 2006; Lechin et al. 1985). However, more recent findings suggest that  $\alpha_2$ -adrenoceptors are overactive in the acute phase of colitis and contribute to a pro-inflammatory milieu, and stimulation of these receptors may exacerbate, whereas their inhibition ameliorates colitis (Bai et al. 2009, 2015; Zádori et al. 2016). These findings raise concern that antihypertensive agents acting at  $I_1$ -IRs and  $\alpha_2$ -adrenoceptors may have detrimental effect in IBD patients.

Because to date (to our best knowledge) no study has been conducted to assess the effect of imidazoline drugs on intestinal inflammation, we aimed to analyse, whether and how moxonidine, rilmenidine and other I<sub>1</sub>-IR ligands influence the course of chemically induced colitis in mice.

## **2. Methods & Materials**

### **2.1. Experimental animals**

Experiments were carried out in 8 weeks old female C57BL/6 mice (National Institute of Oncology, Budapest, Hungary). Animals were housed in a temperature ( $22\pm 2^\circ\text{C}$ )- and humidity-controlled room at a 12-h light/dark cycle under conditions of animal housing and experimentation according to ethical guidelines issued by the Ethical Board of Semmelweis University, based on EC Directive 86/609/EEC. Food and water were available ad libitum.

All procedures conformed to the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes, and all efforts were made to minimize the suffering of animals. Number of animals in each experimental group was reduced to the minimum required for a statistical analysis and preemptive euthanasia was performed if mice became moribund. The experiments were approved by the National Scientific Ethical Committee on Animal Experimentation and permitted by the government (Food Chain Safety and Animal Health Directorate of the Central Agricultural Office (PEI/001/1493-4/2015)).

### **2.2. Induction of colitis**

Colitis was induced by adding DSS (2.5 %, molecular weight: 35000-55 000 Da; TdB Consultancy, Uppsala, Sweden) (Jädert et al. 2014) to the drinking water (tap water) for 3, 5 or 7 days (see Section 2.3). The DSS-containing drinking water was made up fresh every second day to avoid bacterial contamination, and the consumption of water and food was monitored in all groups. Body weight was measured daily during the course of the treatment. The development of colitis was evaluated by recording the disease activity index (DAI) covering the general condition of animals (0, normal; 1, weak, sick in appearance; 2, moribund), stool consistency (0, normal; 1, normal/soft; 2, soft/watery; 3, watery diarrhea) and presence of faecal blood (0, no trace of blood; 1, traces of blood on the stool; 2, bloody stool; 3, gross bleeding). The scores in each category were summed up, resulting in a total score between 0 and 8. At day

7-10 after colitis induction mice were sacrificed by decapitation and their whole colons were removed. The reduction of colon length was used as another parameter to assess colonic inflammation (Jädert et al. 2014). Care was taken not to stretch the colon. The length was expressed as percent of length in the control group. Afterwards full-thickness pieces of the distal colon were excised for further analysis. Serum samples were also collected and stored at -80°C until assayed.

### **2.3. Study design**

*Study 1.* In order to characterize the effect of 2.5 % DSS, 48 mice were randomly allocated into 4 groups. Animals in the 1st group (control, n=12) drank normal tap water for 10 days. Animals in the 2nd (n=6) and 3rd (n=12) groups received 2.5 % DSS for 3 and 5 days, respectively, and were sacrificed on day 10, like the control group (DSS 3d and DSS 5d). Animals in the 4th group received 2.5 % DSS for 7 days (DSS 7d, n=18), but after that they were further divided into 3 subgroups (n=6 in each group) and sacrificed at different time points (on days 7, 9 and 10).

*Study 2.* In 4 separate experiments the effects of moxonidine and rilmenidine (I<sub>1</sub>-IR agonists) as well as of AGN 192403 (highly selective I<sub>1</sub>-IR ligand, putative antagonist) and efaroxan (I<sub>1</sub>-IR antagonist) (Dardonville and Rozas 2004) on the development of colitis were analysed. The chemical names of these drugs and their affinities for IRs and alpha<sub>2</sub>-adrenoceptors are shown in Table 1. In these experiments mice were randomly divided into 5-6 groups (6 mice in each group). The 1st and 2nd groups drank normal tap water and were treated either with saline (SAL) or with moxonidine (MOX, 0.1 mg/kg), rilmenidine (RIL, 0.1 mg/kg), AGN 192403 (AGN, 0.1 mg/kg) or efaroxan (EFA, 1 mg/kg). The other groups drank 2.5 % DSS for 7 days and were treated with either saline (DSS+SAL) or with different doses of the I<sub>1</sub>-IR ligands (moxonidine and rilmenidine: 0.01, 0.1 and 1 mg/kg; AGN 192403: 0.1, 1 and 10 mg/kg; efaroxan: 0.1 and 1 mg/kg). Saline and all test compounds were injected

intraperitoneally (i.p.), once daily, in a volume of 0.1 ml/10 g. The applied doses were selected based on literature data (Cobos-Puc et al. 2009; Gyires et al. 2009; Zeidan et al. 2007; Zhu et al. 1999). All animals were sacrificed at day 9 after colitis induction.

*Study 3.* In this study the effects of two commercially available endogenous IR agonists (agmatine and harmane, Table 1) on the course of DSS-induced colitis were assessed. In 2 experiments 36 and 30 mice were randomly allocated into 6 and 5 groups, respectively (6 mice/group). The 1st and 2nd groups in each experiment drank normal tap water and were treated either with vehicle (VEH; saline in case of agmatine (SAL) or 1.7 % acetic acid in case of harmane), or with agmatine (AGM, 30 mg/kg) or harmane (HAR, 2.5 mg/kg). The other groups drank 2.5 % DSS for 7 days and were treated with either vehicle or with different doses of the endogenous IR ligands (agmatine: 10, 30 and 100 mg/kg; harmane: 2.5 and 10 mg/kg). Agmatine and saline were injected i.p., whereas harmane and 1.7 % acetic acid were injected intragastrically by an oral gavage, in a volume of 0.1 ml/10 g, once daily. The applied doses were selected based on literature data (Aricioglu et al. 2003; Zádori et al. 2014; Zeidan et al. 2007). Animals were sacrificed at day 9 after colitis induction, as in the previous study.

*Study 4.* In this study we analysed whether the effect of IR ligands can be enhanced by injecting them more frequently. 36 mice were divided into 6 groups (6 mice/group). The 1st group drank normal tap water and was treated with saline (SAL). The other 5 groups drank 2.5 % DSS for 7 days and were treated either with saline (DSS+SAL) or with moxonidine (MOX, 0.1 mg/kg), rilmenidine (RIL, 0.1 mg/kg), AGN 192403 (AGN, 0.1 mg/kg) or efaroxan (EFA, 1 mg/kg). Drugs and saline were injected i.p., twice daily (at 9 am and 6 pm). Animals were sacrificed at day 9 after colitis induction.

#### **2.4. Histological analysis**

The distal colon samples were fixed in 40 mg/ml buffered formaldehyde, embedded in paraffin, sectioned (5 µm), and stained with haematoxylin and eosin. Digital micrographs were



taken by an Olympus BX51 microscope and Olympus DP50 camera. The sections were taken randomly from the distal colon, and histological injury was assessed in qualitative terms.

## **2.5. Measurement of colonic myeloperoxidase levels**

The tissue levels of myeloperoxidase (MPO) were determined to quantify inflammation-associated infiltration of neutrophils into the tissue. Full-thickness pieces of the distal colon were excised, shock-frozen in liquid nitrogen and stored at -80°C until assay. After weighing, tissues were homogenized in lysis buffer containing 200 mM NaCl, 5 mM EDTA, 10 mM tris, 10% glycerine and 1 mM PMSF (pH 7.4), supplemented with a protease inhibitor cocktail (cOmplete ULTRA Tablets, Roche, Basel, Switzerland). The MPO content of the supernatant was measured with an enzyme-linked immunosorbent assay (ELISA) kit specific for the mouse protein (Hycult Biotechnology, Uden, the Netherlands). Protein concentration of the homogenates was determined by bicinchoninic acid (BCA) assay kit (Thermo Scientific Pierce Protein Research Products, Rockford, USA) with bovine serum albumin as a standard, and the level of MPO was expressed as ng/mg of total protein.

## **2.6. Measurement of interleukin-6 levels in serum**

Blood samples were allowed to clot at room temperature for 30 min before centrifugation (2000×g, 4°C, 10 min), then sera were collected and stored at -80°C until assay. The interleukin-6 (IL-6) content of sera was determined with ELISA kit specific for the mouse protein (Invitrogen, Thermo Fisher Scientific, Waltham, USA) according the manufacturer's instructions. Protein concentration of samples was determined by BCA assay with bovine serum albumin as a standard, and the level of IL-6 was expressed as pg/mg of total protein.

## **2.7. Materials**

Moxonidine, rilmenidine, AGN 192403 and CYM 5442 (2-(4-(5-(3,4-Diethoxyphenyl)-1,2,4-oxadiazol-3-yl)-2,3-dihydro-1*H*-inden-1-yl amino) ethanol) were ordered from Tocris Bioscience (Bristol, UK). Efaroxan, agmatine, and harmaline, as well as all other chemicals,

unless otherwise stated, were purchased from Sigma (St Louis, MO, USA). For chemical names of IR ligands see Table 1.

## **2.8. Statistical analysis**

Data are expressed as means  $\pm$  S.E.M. Statistical analysis of the data was performed with one-way ANOVA followed by Holm-Sidak post hoc test. Two-way repeated measures ANOVA and Friedman test were employed to compare the time course of weight losses and disease activity indices, respectively, between different groups. Kaplan-Meier test was used for analysis of survival rates. A probability of  $p < 0.05$  was considered statistically significant.

### **3. Results**

#### **3.1. Characterization of the effect of 2.5 % DSS**

Because the severity and pathological features of DSS-induced colitis depend on multiple factors, including the duration of DSS exposure (reviewed e.g. by Perse and Cerar 2012), in the first step we tested different protocols and applied 2.5 % DSS for 3 different time intervals (3, 5 and 7 days). As Fig. 1 shows, DSS induced a typical inflammatory response in all groups, characterized by diarrhea, blood in the faeces, weight loss and colon shortening, and the severity of these symptoms heavily depended on the duration of DSS exposure. Namely, a 3-day exposure to DSS followed by 7 days recovery induced only a mild inflammatory response, which peaked at day 6 and resolved almost completely, whereas a 5-day DSS exposure (followed by 5 days recovery) and a 7-day DSS exposure induced modest and severe colonic inflammations, respectively. Animals in the last group (exposed to DSS for 7 days) were sacrificed at 3 different time points (immediately after DSS exposure, and on days 9 and 10), which allowed us to analyse the time course of colon shortening (Fig. 1C).

Since we hypothesized that IR ligands may have primarily beneficial effect on colonic inflammation, we aimed at inducing moderate to severe colitis and chose the protocol with a 7-day DSS exposure for subsequent studies. Considering the low (60 %) survival rate of animals at day 10 (Fig. 1D) we selected the 9th day as the endpoint of experiments.

#### **3.2. Analysing the effect of various synthetic I<sub>1</sub>-IR ligands on DSS-induced colonic inflammation**

Once-daily administration of I<sub>1</sub>-IR agonists moxonidine (0.01 - 1 mg/kg i.p., Figs. 2A-2C) and rilmenidine (0.01 - 1 mg/kg i.p., Figs. 2D-2F) failed to influence the macroscopic signs (elevation of DAI, weight loss and colon shortening) of DSS-induced colitis. Similar results were obtained when animals were treated with the I<sub>1</sub>-IR antagonists AGN 192403 (0.1 - 10 mg/kg i.p., Figs. 3A-3C) and efaroxan (0.1 - 1 mg/kg i.p., Figs. 3D-3F). Although in case of

AGN 192403 the lowest dose slightly decreased the DAI of DSS-treated mice at day 9 (Fig. 3A), this effect was not dose-dependent. When injected to mice receiving normal tap water, none of the tested compounds affected the investigated parameters (Figs. 2-3).

Qualitative histological analysis of distal colon samples confirmed the macroscopic findings. Compared to the histological picture of non-inflamed colons showing intact crypts and normal mucosal epithelial layer (Fig. 4A), treatment with 2.5 % DSS induced marked inflammation with epithelial destruction, loss of crypts, edematous submucosa, as well as mucosal and submucosal neutrophil infiltration (Fig. 4B), which was not influenced by any of the tested I<sub>1</sub>-IR ligands (Figs. 4C-4F).

Similarly, as Fig. 5A demonstrates, the severe inflammation evoked by DSS was accompanied by remarkable (around 100-fold) elevation of MPO levels in the distal colon, indicating a massive infiltration of neutrophils into the tissue, but none of the I<sub>1</sub>-IR agonists and antagonists had any significant effect on it. Finally, serum samples from mice treated with saline, rilmenidine (0.1 mg/kg) or AGN 192403 (0.1 mg/kg) were analysed for their content of IL-6. The level of this pro-inflammatory cytokine was below the level of detection in control animals and raised upon DSS treatment, but remained unchanged in response to rilmenidine and AGN 192403 (Fig. 5B).

### **3.3. Analysing the effect of endogenous IR ligands on DSS-induced colonic inflammation**

In the next step we analysed the effect of two endogenous IR ligands, agmatine (10 - 100 mg/kg i.p.) and harmane (2.5 - 10 mg/kg per os), administered once daily. As Fig. 6 shows, we obtained similar results as with the synthetic compounds, i.e. they failed to significantly influence the development of DSS-induced colitis. Although in case of 30 mg/kg agmatine we recorded slightly lower DAI values at day 9 than in the DSS control group (Fig. 6A), this effect was not dose-dependent and agmatine did not affect any other inflammatory parameters (Figs.

6B-6C). Neither agmatine, nor harmine (or its vehicle) caused any effect in mice receiving normal tap water.

### **3.4. Analysing the effect of twice-daily administered synthetic I<sub>1</sub>-IR ligands on DSS-induced colonic inflammation**

We wondered whether the effect of I<sub>1</sub>-IR ligands on colitis can be enhanced by changing the treatment regimen and injecting them i.p., twice per day. As Fig. 7 demonstrates, DSS-induced inflammation remained unchanged in response to rilmenidine (0.1 mg/kg), AGN 192403 (0.1 mg/kg) and efaroxan (1 mg/kg). Likewise, moxonidine (0.1 mg/kg) failed to significantly influence the DSS-evoked elevation of DAI and colon shortening, but it slightly enhanced the weight loss of animals (Fig. 7B), which was not due to any effect on their daily food and water consumption (not shown).

#### 4. Discussion

This study demonstrates for the first time that I<sub>1</sub>-IR agonists moxonidine and rilmenidine, similarly to other synthetic and endogenous IR ligands, do not have a major influence on the inflammatory parameters of DSS-induced acute colitis in mice. Although our results suggest that imidazoline antihypertensives have neither significant beneficial, nor detrimental effect in patients with intestinal inflammation, the translational value of these findings needs to be verified with other experimental colitis models and human studies.

Since the recognition and pharmacological characterization of non-adrenergic imidazoline binding sites a substantial effort has been put into understanding their functions and assessing their physiological importance. The I<sub>1</sub>-IR subtype, which displays high affinity for 2-aminoimidazolines such as clonidine, and medium affinity for imidazolines such as idazoxan (Dardonville and Rozas, 2004), has attracted particular attention due to its role in the regulation of cardiovascular functions (Bousquet et al. 1984, 2003). The concept that I<sub>1</sub>-IRs localized in the rostral ventrolateral medulla mediate or at least contribute to the hypotensive effect of clonidine and its structural analogs, whereas alpha<sub>2</sub>-adrenoceptors mediate the adverse effects (sedation, dry mouth) has led to the development of moxonidine and rilmenidine, which possess higher affinity for I<sub>1</sub>-IRs than for alpha<sub>2</sub>-adrenoceptors and used world-wide as antihypertensive agents (Bousquet et al. 2003; Edwards et al. 2012; Verbeuren et al. 1990).

IRs are also localized throughout the GI tract; they were shown on parietal cells (Houi et al. 1987), stomach membranes (Molderings et al. 1995, 1998), gastric and intestinal smooth muscles (Tesson et al. 1992; Wikberg et al. 1991) as well as on colon epithelial cells (Senard et al. 1990). Hence, from the 90's several studies have been conducted to assess the GI effects of imidazoline drugs and the contribution of IRs to these actions. Although the results are sometimes conflicting, the majority of studies indicate that IRs may mediate the stimulatory effect of imidazolines on gastric acid secretion (Glavin et al. 1995; Houi et al. 1987; Molderings

et al. 1999), whereas other receptors, mainly  $\alpha_2$ -adrenoceptors mediate their inhibitory effect on GI contractility and peristalsis (Colucci et al. 1998; Liu and Coupar 1997; Zádori et al. 2013).

There is some direct and indirect evidence that moxonidine and rilmenidine, as well as other imidazoline drugs may have anti-inflammatory properties as well. Rilmenidine and clonidine were shown to reduce acute paw edema induced by carrageenan-, formalin-, serotonin or histamine in rats, an effect mediated most likely by  $\alpha_2$ -adrenoceptors (Gyires et al. 2009; Holsapple et al. 1984; Kulkarni et al. 1986). Moxonidine reduced systemic low-grade inflammation in hypertensive postmenopausal overweight women (Pöyhönen-Alho et al. 2008). Idazoxan and agmatine inhibited the expression of inducible nitric oxide synthase *in vitro* (Regunathan et al. 1999), although agmatine is also capable to interact with multiple other molecular targets, which can contribute to its cyto- and organoprotective effects (reviewed recently by Piletz et al. 2013). Furthermore, various novel imidazoline scaffolds were shown to inhibit human proteasome (Lansdell et al. 2013), which in turn prevented NF- $\kappa$ B mediated gene transcription in cell cultures (Sharma et al. 2004; Kahlon et al. 2009) and the production of pro-inflammatory cytokines tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-6 (IL-6) in human blood (Kahlon et al. 2009). Although in the majority of these studies other molecular targets than IRs were found to mediate the anti-inflammatory action of imidazoline drugs, the intriguing hypothesis of Molderings et al. (2007a, b) that  $I_1$ -IRs may belong to the family of S1P receptors and represent mixtures of homo and/or heterodimers of S1P<sub>1-3</sub> receptors suggests that also pharmacological modulation of  $I_1$ -IRs may result in reduced inflammation. Namely, it is well-established that S1P<sub>1-3</sub> receptors are essential for immune cell trafficking and lymphocyte egress from lymphoid organs (Aoki et al. 2016), and S1P receptor modulators ameliorate the severity of inflammation in different diseases, including colitis (Daniel et al. 2007; Deguchi et al. 2006; Snider et al. 2009). Therefore, it can be hypothesized that  $I_1$ -IR

ligands may interfere with normal SIP signaling, which in turn might induce anti-inflammatory action. All the above data suggest that imidazoline drugs may have beneficial effects on gut inflammation, which could be exploited in the treatment of patients with IBD.

On the other hand, it is well-established that many IR ligands, including moxonidine and rilmenidine, bind with nanomolar affinity to  $\alpha_2$ -adrenoceptors as well (Szabó 2002). Although some data suggest that activation of these receptors by clonidine improve the symptoms of patients with IBD (Furlan et al. 2006; Lechin et al. 1985), more recent evidence indicate that stimulation of  $\alpha_2$ -adrenoceptors may rather exacerbate colitis by inducing directly the release of various pro-inflammatory cytokines and chemokines from immune cells (Bai et al. 2009, 2015; Zádori et al. 2016). Moreover, their activation may inhibit the release of acetylcholine from cholinergic enteric nerves, which otherwise is anti-inflammatory by activating  $\alpha_7$  nicotinic ACh receptors on macrophages (Wang et al. 2003).

Thus, imidazoline drugs can potentially influence colitis in both way; either reduce the severity of inflammation and tissue damage by IR-dependent and/or -independent mechanisms, or exacerbate it by acting on  $\alpha_2$ -adrenoceptors. Either is the case, such an effect could be clinically relevant as well, and should be considered e.g. by the anti-hypertensive treatment of patients with coexisting IBD. Interestingly, to date no study has been conducted to analyse their effect on gut inflammation.

In the present study we induced colitis by adding DSS to the drinking water of mice, which is one of the most commonly used model of intestinal inflammation (Perse and Cerar 2012). Since we hypothesized that IR ligands may have primarily beneficial effect on colonic inflammation, we aimed at inducing moderate to severe colitis, yet with low rate of mortality, and established the appropriate DSS protocol first, in which mice received 2.5 % DSS for 7 days and were sacrificed on day 9. With this protocol, DSS induced severe diarrhea, rectal bleeding and weight loss, which were accompanied by typical histopathological signs of



inflammation and tissue damage, significant reduction of colon length and elevation of MPO and IL-6 levels measured in the colon wall and serum, respectively. Mice were treated once daily with different IR ligands, such as moxonidine and rilmenidine (I<sub>1</sub>-IR agonists), AGN 192403 (highly selective neutral antagonist (Mukaddam-Daher et al. 2006) or partial agonist (Zádori et al. 2014) of I<sub>1</sub>-IRs), efaroxan (I<sub>1</sub>-IR antagonist), as well as agmatine and harmane (endogenous IR agonists). All applied doses were selected based on literature data (see references in Section 2), and the dose range of moxonidine and rilmenidine was also clinically relevant (0.01 - 1 mg/kg, which corresponds approximately to 0.06 - 5.6 mg for a person weighing 70 kg) (Reagan-Show et al. 2007).

By using the above-described DSS protocol and drug dosing regimen, none of the tested IR ligands influenced significantly the macroscopic and histological signs of DSS-induced inflammation. Likewise, the elevated colonic MPO content and serum IL-6 level remained unchanged in response to treatment with these drugs.

Because plasma half-lives of the used I<sub>1</sub>-IR ligands are relatively short (e.g. 2 and 8 hours in case of moxonidine and rilmenidine, respectively) (Edwards et al. 2012; Verbeuren et al. 1990), it might be argued that once daily administration of these agents is not enough to explore their potential anti- or pro-inflammatory actions. Although short plasma half-life does not necessarily mean short duration of action, for example, the antihypertensive action of both moxonidine and rilmenidine lasts 12-24 hours in humans (Edwards et al. 2012; Verbeuren et al. 1990), we changed the treatment regimen and injected them twice daily to test, whether it would enhance their potential intestinal effects. Rilmenidine, AGN 192403 and efaroxan still failed to influence the macroscopic signs of DSS-evoked colitis. Moxonidine had no effect on DAI values and colon shortening either, but it induced a slight weight loss, compared to the DSS control group. Because it did not affect the daily food and water consumption of mice, the wasting of animals may reflect a mild aggravation of disease severity. Since both moxonidine

and rilmenidine behave as agonists at  $\alpha_2$ -adrenoceptors and bind to them with comparable affinities (Eglen et al. 1998; Head and Mayorov 2006; Szabó 2002), but moxonidine is more potent at activating them (Zhu et al. 1999), it is possible that twice daily injection of 0.1 mg moxonidine to mice (which equals approximately to twice daily injection of the maximal recommended daily dose, 0.6 mg, in humans) (Farsang 2001) was capable to induce a slight,  $\alpha_2$ -adrenoceptor-mediated exacerbation of colonic inflammation.

In summary, from our study the following conclusions can be drawn. 1)  $I_1$ -IRs do not influence the severity of acute experimental colitis induced by DSS in the mouse. 2) Although IR ligands might induce anti-inflammatory effects in certain models and diseases, by acting on  $I_1$ -IRs or other molecular targets, this is probably not sufficient to alleviate inflammation of the gut. 3) IR ligands, though bind also to  $\alpha_2$ -adrenoceptors, which may mediate a pro-inflammatory action, failed to aggravate significantly DSS-colitis. Hence, these results may suggest that imidazoline antihypertensives have neither significant beneficial, nor detrimental effect in patients with IBD.

However, it should be emphasized that IR ligands have been tested only in one of the numerous animal models of IBD, and only in the acute phase of inflammation. To date more than 60 different colitis models have been developed, which differ in several aspects, such as pathomechanism, clinical/histological picture and cytokine profile (Hoffmann et al. 2002; Mizoguchi 2012; Wirtz and Neurath 2007). Consequently, a drug maybe effective in one, but ineffective in other model of intestinal inflammation depending on its mechanism of action (Park et al. 2004). Moreover, although DSS-induced colitis is one of the most widely used IBD models, due to the convenient induction, defined onset of intestinal inflammation, and its relevance for the translation of animal data to human IBD (Melgar et al. 2008), even the clinically widely used, effective drugs, such as olsalazine, budesonide or methotrexate have had variable positive effects in this model, depending on several factors (concentration of DSS and

duration of exposure, route of drug administration, genetic background of animals, etc.) (see e.g. Loher et al. 2003; Melgar et al. 2008; Sann et al. 2013). Therefore, although our study strongly suggests that imidazoline drugs do not have a major impact on intestinal inflammation, the translational value of these findings needs to be verified by other experimental colitis models (e.g. by using other chemical inducers or transgenic mice prone to develop colitis) and also by clinical studies.

### **Acknowledgements**

The authors wish to express their thanks to Veronika Pol-Maruzs and Anikó Perkecz for their technical assistance. This work was supported by the Hungarian Scientific Research Fund [OTKA PD 109602].

The authors declare that they have no conflict of interest.

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## Figure legends

**Fig. 1.** Characterization of the effect of 2.5 % dextran sulfate sodium (DSS) by using different protocols. DSS was applied for 3 different time intervals (3, 5 and 7 days), control animals drank normal tap water. Animals receiving DSS for 3 days (n=6) and for 5 days (n=12), as well as control animals (n=12) were sacrificed on day 10, whereas animals receiving DSS for 7 days (n=18) were sacrificed at 3 different time points (6-6 mice on days 7, 9 and 10). The severity of colitis was assessed by determining the disease activity index (DAI) (A), weight loss (B), reduction of colon length (C) and survival rate (D). The values represent means  $\pm$  SEM. \* $p < 0.05$  vs control group, for statistical analysis Friedman test (panel A), two-way repeated measures ANOVA (panel B) and one-way ANOVA (panel C) were used, followed by Holm-Sidak post hoc test. Kaplan-Meier test was used for analysis of survival rates (panel D).

**Fig. 2.** The effect of moxonidine (MOX, 0.01 - 1 mg/kg, panels A-C) and rilmenidine (RIL, 0.01 - 1 mg/kg, panels D-F) on the macroscopic signs of DSS-induced colitis. Drugs or saline (SAL) were injected intraperitoneally (i.p.), once daily. Neither moxonidine, nor rilmenidine influenced significantly the DSS-evoked elevation of disease activity index (DAI), weight loss or colon shortening. The values represent means  $\pm$  SEM (n=6/group). \* $p < 0.05$  vs SAL. For statistical analysis Friedman test (panels A, D), two-way repeated measures ANOVA (panels B, E) and one-way ANOVA (panels C, F) were used, followed by Holm-Sidak post hoc test.

**Fig. 3.** The effect of AGN 192403 (AGN, 0.1 - 10 mg/kg, panels A-C) and efaroxan (EFA, 0.1 - 1 mg/kg, panels D-F) on the macroscopic signs of DSS-induced colitis. Drugs or saline (SAL) were injected intraperitoneally (i.p.), once daily. Neither AGN 192403, nor efaroxan had major impact on DSS-evoked elevation of disease activity index (DAI), weight loss or colon shortening. The values represent means  $\pm$  SEM (n=6/group). \* $p < 0.05$  vs SAL, <sup>+</sup> $p < 0.05$  vs DSS+SAL. For statistical analysis Friedman test (panels A, D), two-way repeated measures

ANOVA (panels B, E) and one-way ANOVA (panels C, F) were used, followed by Holm-Sidak post hoc test.

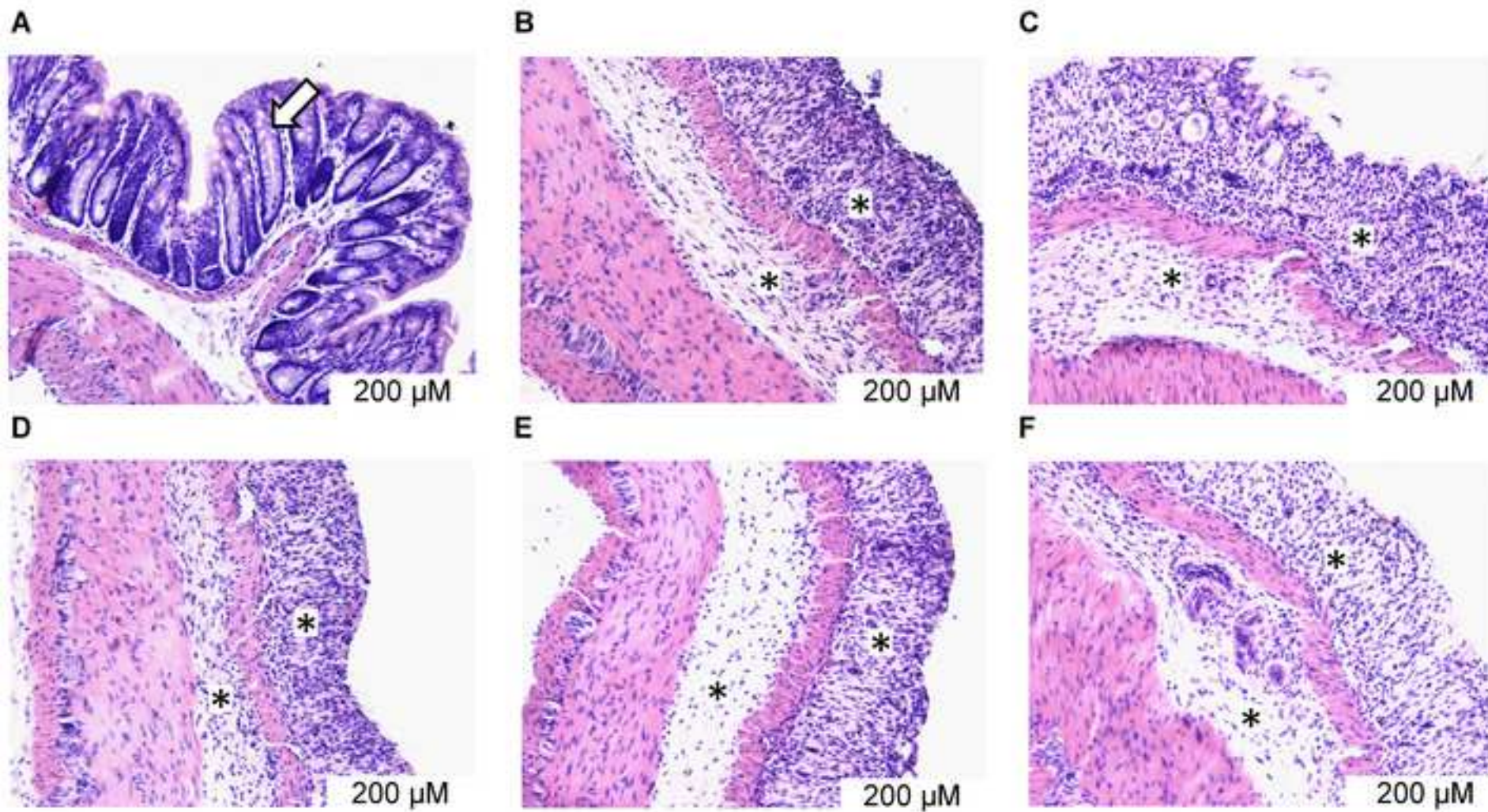
**Fig. 4.** Representative histological micrographs of colonic tissue sections, haematoxylin-eosin staining. Full-thickness pieces of the distal colon were obtained from mice treated with saline i.p. + normal tap water (A), saline i.p. + 2.5 % DSS (B), moxonidine 0.1 mg/kg i.p. + 2.5 % DSS (C), rilmenidine 0.1 mg/kg i.p. + 2.5 % DSS (D), AGN 192403 0.1 mg/kg i.p. + 2.5 % DSS (E) or efaroxan 1 mg/kg i.p. + 2.5 % DSS (F). Histological signs of severe inflammation were observed in all DSS-treated groups, irrespectively of whether they were treated with I<sub>1</sub>-IR ligands or not. Arrow shows intact crypts in control group, whereas asterisks show massive mucosal and submucosal neutrophil infiltration in DSS-treated groups. Scalebar: 200 µm.

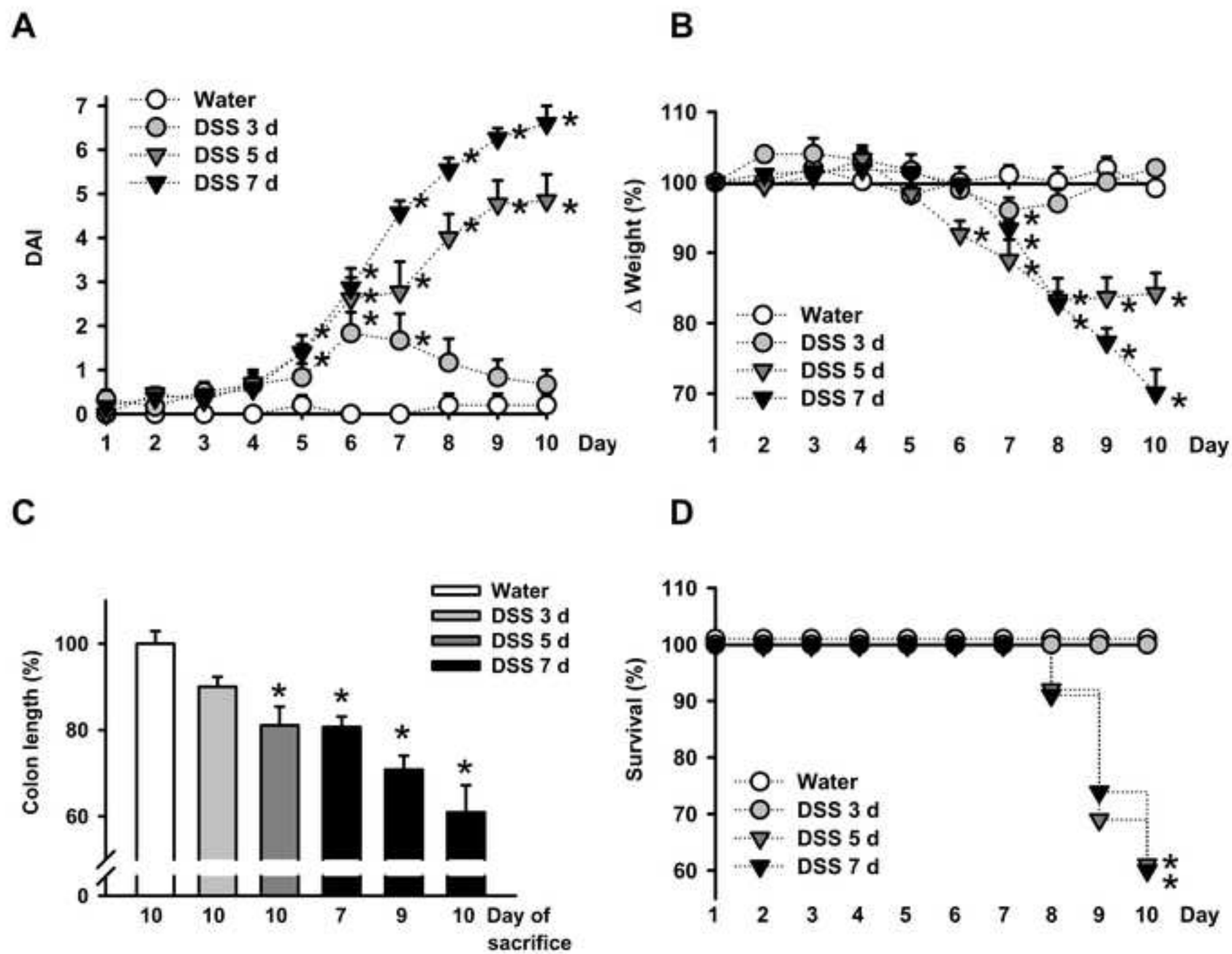
**Fig. 5.** The effect of synthetic I<sub>1</sub>-IR agonists and antagonists on DSS-induced elevation of colonic myeloperoxidase (MPO) and serum interleukin-6 (IL-6) levels. Panel A: Moxonidine (MOX, 0.1 mg/kg), rilmenidine (RIL, 0.01 - 1 mg/kg), AGN 192403 (AGN, 0.1 - 10 mg/kg) and efaroxan (EFA, 1 mg/kg) all failed to significantly influence the DSS-evoked elevation of MPO level in the colon wall. The values represent means ± SEM (DSS+SAL group: n=13, all other groups: n=6). \*p<0.05 vs SAL, one-way ANOVA, followed by Holm-Sidak post hoc test. Panel B: Rilmenidine (RIL, 0.1 mg/kg) and AGN 192403 (AGN, 0.1 mg/kg) did not influence the serum IL-6 content of mice, compared to DSS+SAL group. The values represent means ± SEM (n=5/group). One-way ANOVA, followed by Holm-Sidak post hoc test was used for statistical analysis between DSS-treated groups. Serum IL-6 level in the control group (saline i.p. + tap water) was below the level of detection limit (ND, not detectable).

**Fig. 6.** The effect of agmatine (AGM, 10 - 100 mg/kg, panels A-C) and harmane (HAR, 2.5 - 10 mg/kg, panels D-F) on the macroscopic signs of DSS-induced colitis. Agmatine and saline (SAL) were injected i.p., whereas harmane and its vehicle (VEH), 1.7 % acetic acid were injected intragastrically by an oral gavage, once daily. Neither agmatine, nor harmane had major

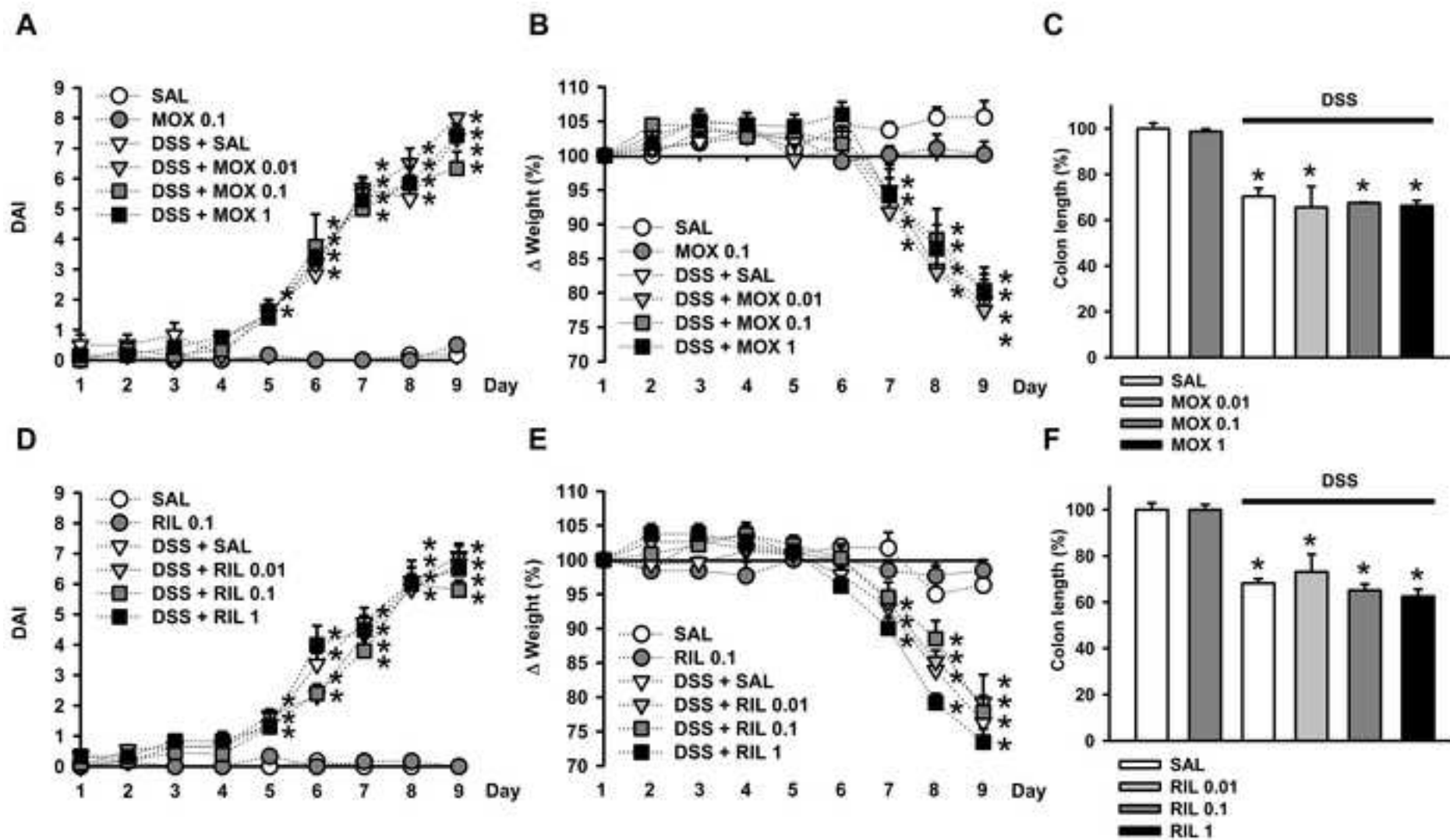
effect on DSS-evoked elevation of disease activity index (DAI), weight loss or colon shortening. The values represent means  $\pm$  SEM (n=6/group). \*p<0.05 vs SAL, <sup>+</sup>p<0.05 vs DSS+SAL. For statistical analysis Friedman test (panels A, D), two-way repeated measures ANOVA (panels B, E) and one-way ANOVA (panels C, F) were used, followed by Holm-Sidak post hoc test.

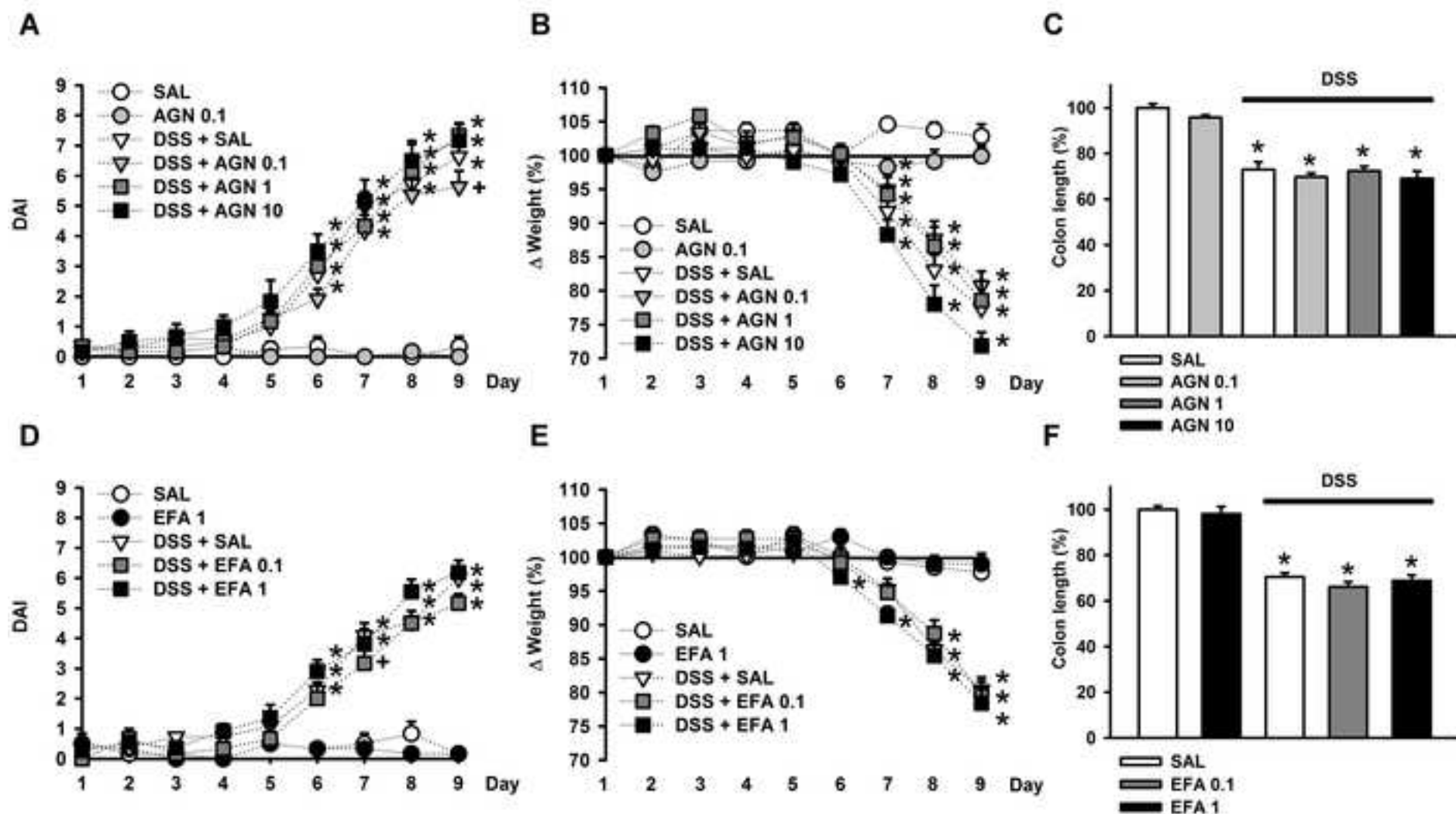
**Fig. 7.** The effect of twice-daily administered synthetic I<sub>1</sub>-IR agonists and antagonists on the macroscopic signs of DSS-induced colitis. Moxonidine (MOX, 0.1 mg/kg), rilmenidine (RIL, 0.1 mg/kg), AGN 192403 (AGN, 0.1 mg/kg) and efaroxan (EFA, 1 mg/kg), as well as saline (SAL) were injected i.p. Moxonidine slightly aggravated the DSS-induced weight loss, whereas the other drugs failed to affect the DSS-induced elevation of disease activity index (DAI) (A), weight loss (B) or colon shortening (C). The values represent means  $\pm$  SEM (n=6/group). \*p<0.05 vs SAL, <sup>+</sup>p<0.05 vs DSS+SAL. For statistical analysis Friedman test (panel A), two-way repeated measures ANOVA (panel B) and one-way ANOVA (panel C) were used, followed by Holm-Sidak post hoc test.

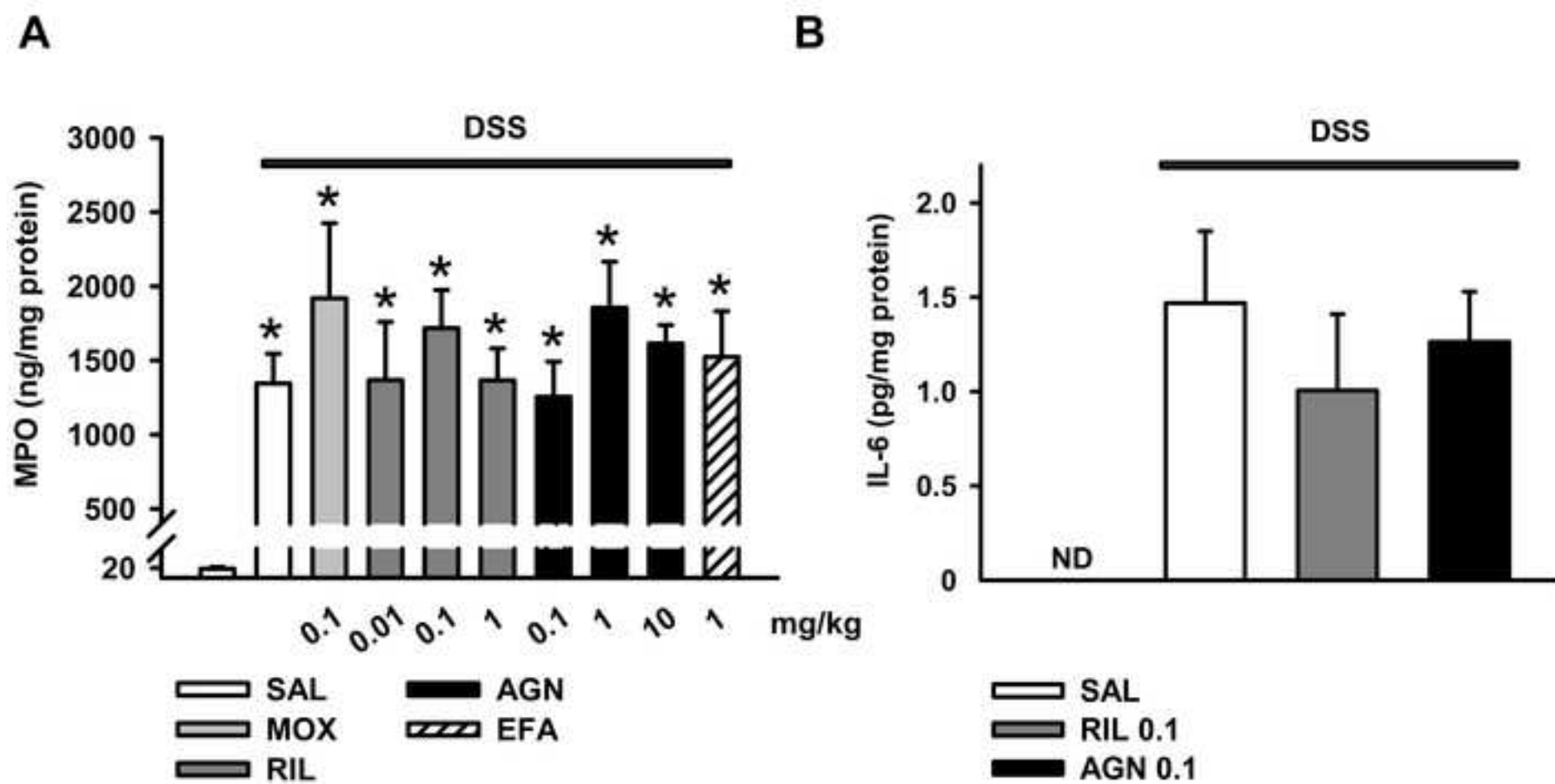


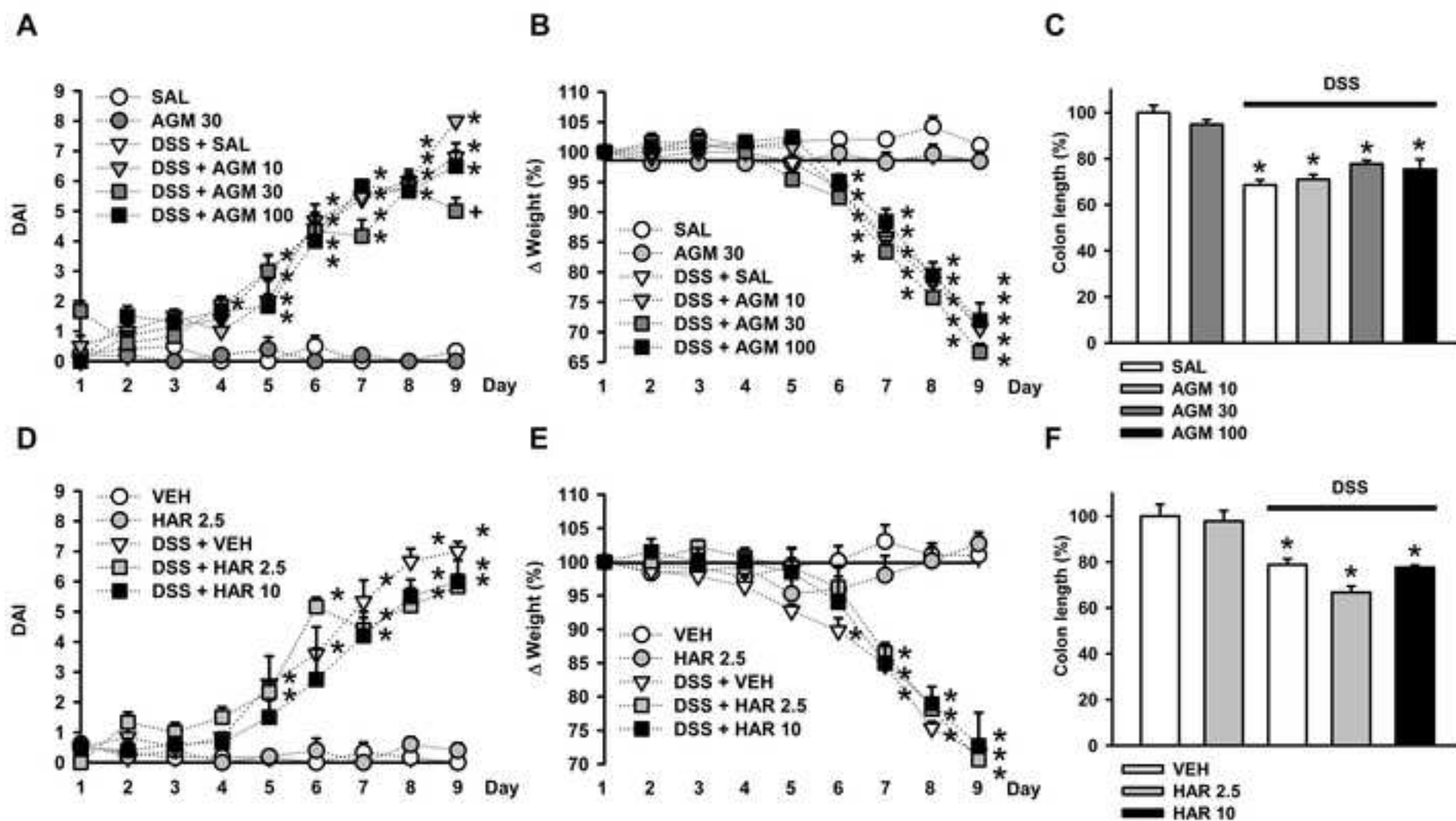


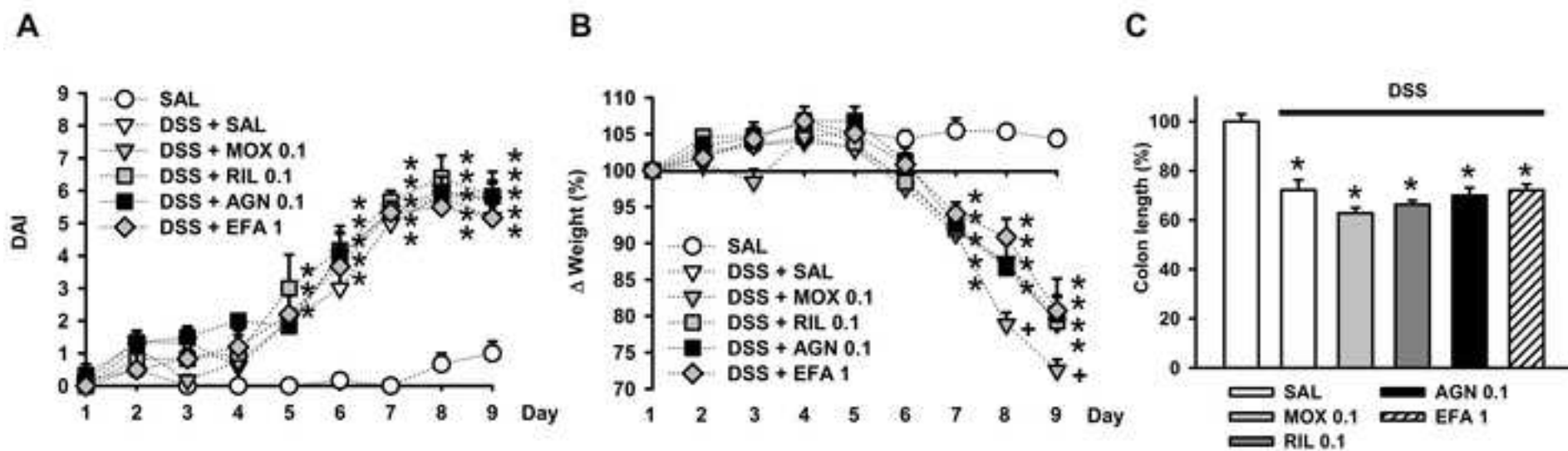












**Table 1. Chemical names of tested IR ligands and their affinity constants (pKi) in various cells and tissues for I<sub>1</sub>- and I<sub>2</sub>-IRs, and to alpha<sub>2</sub>-adrenoceptors (alpha<sub>2</sub>-AR)**

Drug	Chemical name	I <sub>1</sub> -IR (pKi)	I <sub>2</sub> -IR (pKi)	alpha <sub>2</sub> -AR (pKi)
Moxonidine	4-Chloro- <i>N</i> -(4,5-dihydro-1 <i>H</i> -imidazol-2-yl)- 6-methoxy-2-methyl-5-pyrimidinamine	8.37 <sup>a</sup>	<5 <sup>a</sup>	7.11 <sup>a</sup>
Rilmenidine	2-[ <i>N</i> -(Dicyclopropylmethyl)amino] oxazoline	7.22 <sup>a</sup>	5.96 <sup>a</sup>	6.90 <sup>a</sup>
AGN 192403	(±)-2-endo-Amino-3-exo-isopropylbicyclo [2.2.1] heptane	7.38 <sup>b</sup>	ND <sup>b</sup>	<5 <sup>b</sup>
Efaroxan	2-ethyl-2-(imidazolin-2-yl)-2,3- dihydrobenzofuran	7.28 <sup>a</sup>	<5 <sup>a</sup>	5.74-7.87 <sup>a</sup>
Agmatine	(4-Aminobutyl)guanidine	7.48 <sup>a</sup>	<5 <sup>a</sup>	<5 <sup>a</sup>
Harmane	1-Methyl-9H-pyrido[3,4- <i>b</i> ]indole	7.51 <sup>c</sup>	7.31 <sup>c</sup>	<5 <sup>c</sup>

Ki values were taken from the following references: <sup>a</sup>Eglen et al. 1998; <sup>b</sup>Munk et al. 1996; <sup>c</sup>Husbands et al. 2001. (Affinity values, however, can vary substantially in different species and tissues.) Affinities for non-I<sub>1</sub>-non-I<sub>2</sub>-IRs are in most cases lacking, and are not included in the table.